

# Human herpesvirus-6, varicella-zoster virus and oligoclonal bands in demyelinating diseases of the central nervous system

Jenna Nicklén

Doctoral Programme in Clinical Research

Department of Virology, Haartman Institute, Medicum

Department of Neurology, Clinicum

Faculty of Medicine

University of Helsinki

Finland

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**Supervisor**

Professor Markus Färkkilä  
Department of Neurology, Clinicum  
Faculty of Medicine  
University of Helsinki

**Reviewers**

Docent Merja Soilu-Hänninen  
Department of Neurology  
Faculty of Medicine  
University of Turku

Docent Veijo Hukkanen  
Department of Virology  
Faculty of Medicine  
University of Turku

**Official Opponent**

Professor Irina Elovaara  
Department of Neurology  
Faculty of Medicine  
University of Tampere

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## SUPPLEMENT

“Potilaskaavake”

## LIST OF ORIGINAL PUBLICATIONS

- I** Pietiläinen J, Virtanen JO, Uotila L, Salonen O, Koskiniemi M and Färkkilä M. HHV-6 infection in multiple sclerosis. A clinical and laboratory analysis. *Eur J Neurol.* 2010;17(3):506-9.
- II** Virtanen JO\*, Pietiläinen-Nicklén J\*, Uotila L, Färkkilä M, Vaheri A, Koskiniemi M. Intrathecal human herpesvirus 6 antibodies in multiple sclerosis and other demyelinating diseases presenting as oligoclonal bands in cerebrospinal fluid. *J Neuroimmunol.* 2011;15;237(1-2):93-7.
- III** Pietiläinen-Nicklén J, Virtanen JO, Uotila L, Salonen O, Färkkilä M, Koskiniemi M. HHV-6-positivity in diseases with demyelination. *J Clin Virol.* 2014;61(2):216-9.
- IV** Pietiläinen-Nicklén J, Virtanen O, Kolehmainen P, Uotila L, Salonen O, Lappalainen M, Färkkilä M, Koskiniemi M. ADEM and virus infection. *J Clin Virol.* 2012;53(1):2-5.
- V** Pietiläinen-Nicklén J, Kolehmainen P, Blom T, Uotila L, Kankaanpää A, Virtanen JO, Färkkilä M. Varicella zoster virus -reactive oligoclonal bands in patients with demyelinating disease. Manuscript.

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## ABBREVIATIONS

BAEP	brainstem auditory evoked potential
BBB	blood-brain barrier
CDMS	clinically definite multiple sclerosis
CFS	chronic fatigue syndrome
CIS	clinically isolated syndrome
CIHHV-6	chromosomally integrated human herpesvirus-6
CNS	central nervous system
CPMS	clinically probable multiple sclerosis
CRP	C-reactive protein
CSF	cerebrospinal fluid
DC	dendritic cell
EBV	Epstein-Barr virus
EDSS	expanded disability status scale
ES	exanthema subitum
HHV-6	human herpesvirus-6
IEF	isoelectric focusing
JCV	John Cunningham virus, JC-virus
MBP	myelin basic protein
MOG	myelin oligodendrocytic glycoprotein
MS	multiple sclerosis
OCB, OCBs	oligoclonal band, oligoclonal bands
PB	peripheral blood
PLP	proteolipid protein
PPMS	primary progressive multiple sclerosis
RBC	red blood cell

RIS	radiologically isolated syndrome
RRMS	relapsing-remitting multiple sclerosis
SEP	somatosensory evoked potential
SPMS	secondary progressive multiple sclerosis
Treg	regulatory T cells
VEP	visual evoked potential
VZV	varicella-zoster virus
WBC	white blood cell

## ABSTRACT

**Background** Multiple sclerosis (MS) is an autoimmune disease of the central nervous system (CNS) whereby the host immune system attacks against the myelin. MS affects predominantly young adults and leads to neurological disability. Although it has worldwide penetrance, MS has different incidence rates in different parts of the world. The incidence may also vary among different parts of the country, as seen in Finland. The final diagnosis of MS may often be delayed due to the heterogeneity and relapsing nature of the disease. Occurring symptoms depend on the location of the inflammation in the central nervous system. The most common form of the disease is relapsing-remitting multiple sclerosis (RRMS), in which patients typically recover from all of their symptoms. Infections are often seen before the disease progresses or relapses. Therefore, infectious agents, especially viruses, have been under suspicion for triggering an autoimmune reaction that leads to demyelination. Human herpesviruses are considered to be possible triggers for MS pathogenesis.

**Objective** Most patients who have been diagnosed with MS have oligoclonal bands (OCBs) of immunoglobulins in their cerebrospinal fluid (CSF). The OCBs are intrathecally produced antibodies mainly consisting of IgG, IgA, and IgM class antibodies. Clinically, the most important antibody group in OCB analysis is the IgG class, which was the main focus in this study. The objective was to study if the OCBs of patients contain antibodies against highly neurotropic and common childhood viruses: human herpesvirus-6 (HHV-6) and varicella-zoster virus (VZV). Another objective was to study if the patients, who have virus-reactive OCBs in their CSF, have some distinguishable features. The main aim was to study the possible role of these viruses in the pathogenesis of MS, focusing on the presence of antibodies during the early stages of the disease.

**Methods** OCB-positive CSF-serum sample pairs were systematically collected over the course of one year. A retrospective re-detection of OCBs by isoelectric focusing (IEF) was made, and HHV-6A, HHV-6B and VZV-reactive OCBs were localized with affinity driven immunoblotting.



HHV-6 IgG antibodies were analyzed from the serum with immunofluorescence assay (IFA). The binding capacity of the IgG antibodies was analyzed and the infections (primary vs. past infection) were classified with avidity testing.

During the clinical evaluation, the medical records of the patients were analyzed without knowing the results of the IFA or virus-reactive OCBs. This study has been ethically approved.

**Results** We had 18 immunocompetent adult patients with serologically primary HHV-6A or HHV-6B infections. None of them had any typical signs of a virus infection (e.g. fever or rash). Of those 18 patients 11 were diagnosed with MS with a primary infection during the early stage of the disease.

Of 79 patients, 26 had HHV-6A-, HHV-6B-, or VZV –reactive OCBs in their CSF. Of those 26 patients 62% were diagnosed with MS and had these virus-reactive OCBs during the early stage of MS. Patients who had any studied virus-reactive OCBs in their CSF seemed to differ from those without, including: more OCBs ( $p=0.001-0.003$ ), lower protein concentration ( $p=0.012$ ), and higher IgG index ( $p=0.007-0.014$ ) in the CSF. They were also younger ( $p=0.047$ ).

**Conclusion** Virus-reactive OCBs are possibly associated with MS disease pathogenesis. HHV-6 and VZV may have some association with MS disease. The pathogenesis of multiple sclerosis may have distinguishable subgroups, including pathogenesis triggered by infections with different viruses.

## TIIVISTELMÄ

### Lähtökohdat

Multippeliskleroosi eli MS-tauti on keskushermoston autoimmuunitauti, jossa potilaan oma immuunijärjestelmä tuhoaa ensisijaisesti keskushermoston hermotukikudosta, myeliiniä. MS-tauti on yksi yleisimmistä työkyvyttömyyttä aiheuttavista nuorten aikuisten neurologisista taudeista. Tautia tavataan kaikkialla maailmassa, mutta sen ilmaantuvuus vaihtelee. Vaihtelua on jopa maan sisällä, kuten nähdään myös Suomessa. Diagnoosin varmistuminen voi joskus viivästyä, sillä oireet ja taudinkuva voivat olla joillakin potilailla hyvinkin vaihtelevia. Potilaan oireisto määräytyy sen mukaan missä kohtaa keskushermostoa tulehdus sijaitsee. Tyypillisin taudin ilmentymä on relapsoiva-remittoiva MS-tauti (RRMS), jossa vaihtelevat oirejaksot ja oireettomat jaksot. Erilaisia tulehduksia nähdään usein ennen MS-taudin puhkeamista ja ennen oirejaksoja. Tämän vuoksi etenkin virusten, ja erityisesti ihmisen herpesvirusten, on ajateltu liittyvän MS-taudin puhkeamiseen.

**Tavoitteet** Suurimmalla osalla MS-tautia sairastavista potilaista nähdään aivo-selkäydinnesteessä keskushermostoperäisiä IgG-luokan vasta-aineita oligoklonaalisisina fraktioina (OCB). Oligoklonaalisia fraktioita voidaan nähdä myös IgM- sekä IgA-luokan vasta-aineilla. Ainakin toistaiseksi IgG-luokan OCB:t ovat ainoita, jotka ovat kliinisessä käytössä ja näin ollen ainoastaan näitä tarkastelimme tutkimukssamme. Tutkimuksemme yhtenä tarkoituksena oli selvittää onko osa potilaiden OCB:sta ihmisen herpesvirus-6:n (HHV-6) tai vesirokkoviruksen (VZV) vasta-aineita. Toisena tarkoituksena oli selvittää onko näillä potilailla, joilla osa OCB:sta on virusperäisiä vasta-aineita, joitakin erityispiirteitä. Tutkimuksen varsinaisena tavoitteena oli tutkia virusten osuutta MS-taudin patogeneesissä ja keskittyä erityisesti tarkastelemaan edellä mainittuja herpesvirusvasta-aineita taudin varhaisvaiheissa.

## Menetelmät

Keräsimme yhden vuoden ajanjaksolta seerumi- ja aivoselkäydinnestenäytteet kaikilta Meilahden neurologian klinikalla hoidetuilta potilailta, joilla oli keskushermostoperäisiä OCB:ta likvorissa. Teimme retrospektiivisesti kaikille näytteille toistetun isoelektrisen fokusoinnin määrittääksemme OCB:t. Käytimme affiniteetti-immunoblottausta paikantaaksemme oligoklonaalisten fraktioiden sisältämät mahdolliset HHV-6A:n, HHV-6B:n sekä VZV:n vasta-aineet. Seerumin HHV-6 IgG-luokan vasta-aineet määritettiin epäsuoralla immunofluoresenssilla. IgG-luokan vasta-aineen sitoutumisvoimakkuutta analysoimme aviditeetti-testauksella. Näin saimme luokiteltua infektion joko primaari-infektioksi tai aikaisemmin sairastetuksi infektioksi. Potilaiden kliiniset tiedot kerättiin ja analysoitiin sokkoutetusti ilman tietoja laboratoriotuloksista. Tutkimuksella on eettisen toimikunnan hyväksyntä.

**Tulokset** Löysimme yhteensä 18 immunokompetenttia aikuista, joilla oli seerumin vasta-aineiden perusteella HHV-6A tai HHV-6B primaari-infektio. Yhdelläkään näistä potilaista ei ollut tyypillisiä virusinfektion oireita (esim. kuume tai ihottuma). Näistä 18 potilaasta 11:llä diagnosoitiin tuore MS-tauti HHV-6 primaari-infektion aikana. Lisäksi kaikista 79 tutkitusta OCB positiivisesta potilaasta 26:lla oli keskushermostoperäistä HHV-6A:n, HHV-6B:n tai VZV:n vasta-ainetta oligoklonaalisisissa fraktioissa. Näistä 26 potilaasta 62 %:lla oli näitä virusvasta-aineita varhaisen MS-taudin diagnoosin aikana. Potilaat, joilla oli jokin näistä tutkituista keskushermostoperäisistä virusvasta-aineista, näyttivät eroavan niistä potilaista, joilla ei ollut lainkaan tutkittuja virusvasta-aineita. Heillä oli lukumääräisesti enemmän likvorin oligoklonaalisia fraktioita ( $p=0.001-0.003$ ), pienempi likvorin proteiinimäärä ( $p=0.012$ ) ja korkeampi likvorin IgG indeksin luku ( $p=0.007-0.014$ ). He olivat lisäksi nuorempia ( $p=0.047$ ).

**Päätelmät** Oligoklonaalisten fraktioiden sisältämät virusvasta-aineet ovat mahdollisesti yhteydessä MS-taudin patogeneesiin. Ihmisen herpesvirus-6 sekä vesirokkovirus saattavat olla osallisina MS-taudin puhkeamisessa. MS-taudissa on mahdollisesti patogeneettisesti eriäviä ryhmiä, joista osa saattaa selittyä erilaisten virusten aiheuttamilla infektioilla.

## INTRODUCTION

Multiple sclerosis (MS) is one of the most disabling neurological diseases in young adults. It usually occurs in previously healthy individuals without any prodromal signs. Presenting symptoms may vary from mild paresthesia to disabling motoric deficiency and diplopia. Patients may suffer mild recurring symptoms years before being referred to a neurologist. In some cases, it may be extremely challenging to definitively diagnose MS. A curative medication for MS is yet to be found. Some new immunotherapies significantly reduce the relapses and inflammatory activity that is seen in the MRI of the brain. The disease progression can also be moderately delayed with several immunotherapies. MS pathogenesis is dependent on leucocyte-mediated demyelination. Drugs that reduce the number of activated leucocytes are most effective against relapses of MS. The main triggers that cause the immune system to incorrectly attack the host's own myelin are thought to be combinations of genetic predisposition and environmental factors, including viruses.

Viruses and various infections have been associated with MS pathogenesis for nearly a century. Mild infections are usually observed before disease progression and relapse. Human herpesviruses have been most studied and they are thought to have some association with MS pathogenesis. Currently, Epstein-Barr virus (EBV) is the strongest viral candidate in MS pathogenesis and Human herpesvirus-6 is also of significant interest in research. Although many different viruses have been actively researched during the last decades, an "MS-triggering virus" has not been identified.

An immune system dysfunction that causes the immune system to attack itself and cause demyelination is thought to be the main pathological mechanism in MS disease. Clinical symptoms and characteristic white matter lesions are the consequences of the demyelination process in the central nervous system (CNS). The presence of intrathecal immunoglobulin antibodies, called oligoclonal bands (OCBs), is a highly common finding in cerebrospinal fluid (CSF) of the patients. In MS pathogenesis, OCBs are thought to be a sign of immune-system dysfunction and an indicator of the possible involvement of an infectious agent.

The objective of this doctoral study was to investigate the role of viruses during the early stages of MS disease, especially focusing on highly neurotropic ones: human herpesvirus-6A (HHV-6A), human herpesvirus-6B (HHV-6B), and varicella-zoster virus (VZV). HHV-6 and VZV have characteristics that make them highly potential triggers for chronic neurological diseases: I) they both infect nearly all people worldwide and remain in the host for life in a latent form, II) they are

highly neurotropic, especially HHV-6 that is located in the central nervous system in a latent state, and III) they both act as opportunistic pathogens that may be activated when the immune system is compromised.

In our study, we found patients who had serologically confirmed primary HHV-6A/B infections in the early stages of MS. We also found patients with early MS who had HHV-6 –reactive OCBs in their CSF and patients with VZV-reactive OCBs. The prevalence and possible clinical association of these findings were investigated further.

## 1 LITERATURE REVIEW

### 1.1 HUMAN HERPESVIRUS-6 (HHV-6)

#### 1.1.1 *Introduction of the virus*

HHV-6 was first discovered in 1986 in peripheral blood leukocytes of patients with AIDS and patients with lymphoproliferative disorders (Salahuddin et al., 1986). It was first called human B-lymphotropic virus, but later recognized to be a member of the human herpesvirus family and re-named as HHV-6 (Ablashi et al., 2014, Ablashi et al., 1987). HHV-6 is a beta-herpes virus belonging to the herpes virus family along with herpes simplex 1 and 2 (HSV-1 and HSV-2, HHV-1 and HHV-2), varicella-zoster virus (VZV, HHV-3), Epstein-Barr virus (EBV, HHV-4), cytomegalovirus (CMV, HHV-5), HHV-7, and Kaposi's sarcoma associated herpesvirus (KSHV, HHV-8).

HHV-6 was long considered to have two variants: HHV-6A and HHV-6B. These were recently (2012) re-classified by the International Committee on Taxonomy of Viruses as two separate viruses (Ablashi et al., 2014). HHV-6B is known to cause exanthema subitum – a common childhood illness (ES, roseola infantum, sixth disease). The clinical course of ES is typically benign or nearly symptomless and self-limiting. Typical symptoms of ES often include fever that is followed by a characteristic mild rash (see Figure 1). Complications are uncommon and the primary infection is rarely fatal (Campadelli-Fiume et al., 1999). In fulminant cases of exanthema subitum, patients may have febrile seizures or even encephalitis (Laina et al., 2010).



Figure 1. Typical rash in exanthema subitum. (Photograph is published with the permission of Tiina Iisalo)

HHV-6A has not been proven to cause any specific disease, but it is associated with CNS infections and chronic neurological diseases, including MS (Portolani et al., 2005). The primary infection caused by both HHV-6 viruses is often reported to be mainly asymptomatic; in childhood, HHV-6B causes clinical symptoms more often than HHV-6A, which usually leads to a symptomless primary infection that occurs soon after HHV-6B (Agut, 2011).

#### *1.1.2 Characteristic features*

Nearly all people worldwide have had an HHV-6 infection in early childhood. According to data gathered from different countries, the seroprevalence of HHV-6 antibodies is between 60-95% of the whole population (Krueger and Ablashi, 2003). HHV-6 acquisition appears to be 9-21 months (Zerr et al., 2005). After the primary infection, HHV-6 remains in the host in a latent form permanently. It enters a state of latency in a small fraction of the host's cells in different organs, especially in salivary glands, peripheral blood lymphocytes, and bronchial glands (Mori and Yamanishi, 2007, Pellett et al., 2012, Lyall, 1996). One important site for the presence of the latent

form of HHV-6 is the human brain (Chan et al., 2001, Gordon et al., 1996). It also seems to be latent in the ganglia of the peripheral nervous system (Hufner et al., 2007).

HHV-6 is a highly neurotropic virus (Tuke et al., 2004). It is capable of infecting different cell types in the central nervous system (CNS): oligodendrocytes, astrocytes, and neurons (De Bolle et al., 2005). The hiding nature of the virus is associated with its capability of inducing neurological diseases, and its re-activation might be the cause of the symptoms (Yao et al., 2010a). In addition to neurotropism, HHV-6 is a highly lymphotropic virus, and circulating polymorphonuclear leukocytes are the main reservoir of HHV-6 in the blood during the infection (Gautheret-Dejean et al., 2009). In addition, HHV-6 has tropism for a variety of other human cells and tissues, which is seen in the wide variety of organs the virus is capable of infecting (Ablashi et al., 2010).

In the primary infections caused by HHV-6, pathological mechanisms are thought to be different from those acting during the re-activation of the virus (Kawamura et al., 2011). This can be seen when comparing the clinical outcomes of the primary infection (exanthema subitum) with the re-activation in immunosuppressed patients with organ transplants (i.e. pneumonitis, hepatitis, gastrointestinal dysfunctions, and neurological dysfunctions) (Neumann et al., 2009).

Lately, HHV-6 has been found to be able to integrate into the human genome (Tanaka-Taya et al., 2004). The condition is called chromosomally-integrated HHV-6 (CIHHV-6). In CIHHV-6, patients may have HHV-6-DNA present in the serum or the CSF even when an active HHV-6 infection is not present. The clinical significance of CIHHV-6 remains unknown. Many patients are healthy and symptomless with CIHHV-6, but a variety of neurological symptoms have been associated with the condition in some patients (Montoya et al., 2012).

#### *1.1.3 Clinical significance in adults*

HHV-6 is an opportunistic pathogen, which is often re-activated when a patient is immunocompromised (i.e. patients with organ transplant and hematological malignances) and it is capable of causing moderate to severe CNS diseases (Zerr et al., 2002). For the immunocompromised patients, HHV-6 is capable of causing severe encephalitis. The symptoms in HHV-6-caused encephalitis may be indistinguishable from the herpes-simplex virus -encephalitis (Noguchi et al., 2010). Even though immunocompromised patients survive the HHV-6 encephalitis, they may still suffer from sustained neuropsychological disorders (Sakai et al., 2011).



HHV-6 encephalitis as a complication increases the mortality rate in hematopoietic cell transplant patients, even when it is not the main cause of death (Bhanushali et al., 2013). After a hematopoietic cell transplantation, 30-80% of the patients develop (within 6 weeks) HHV-6B viraemia and some patients may experience CNS dysfunction (Hill et al., 2014). The presence of HHV-6B in the CSF of patients after hematopoietic cell transplantation, even without a clinical CNS dysfunction, significantly increases the mortality risk of the patients (Hill et al., 2014).

It is thought that HHV-6 re-activation is clinically unnoticeable unless the patient has an insufficient immune system (Krueger and Ablashi, 2003). In rare cases, however, HHV-6 can infect immunocompetent adults and cause severe infection in the CNS and other organs (i.e. acute liver dysfunction) (Sloots et al., 1995, Sawada et al., 2007, Fried et al., 2009, De Simone et al., 2013, Cacheux et al., 2005).

#### *1.1.4 Diagnostic tools for diagnosing HHV-6 infection in the CNS*

The presence of HHV-6 DNA in the CSF is highly specific for the diagnosis of HHV-6 CNS infection (Sauerbrei and Wutzler, 2002). In HHV-6 CNS infections the levels of the virus are low, and it is recommended that a quantitative real-time PCR should be used to detect HHV-6 DNA from the CSF (Aberle and Puchhammer-Stockl, 2002, Rotola et al., 2004, Gaeta et al., 2009). The genome of the virus is only detectable during the first few days of infection (Linde et al., 1997). Therefore, even though the DNA detection is a highly specific method, the negative result does not exclude an ongoing infection (Sauerbrei and Wutzler, 2002). The method may also have problems in distinguishing CIHHV-6 from a true infection (Caserta et al., 2010).

Virus-specific IgG antibodies, that are present in the serum and the CSF, can be detected with different methods (eg. enzyme-linked immunosorbent assay (ELISA) and immunofluorescent assay (IFA)). In HHV-6 CNS infections, as in other human herpesvirus CNS infections, intrathecally produced IgG antibodies are detectable in the post-acute stage of the infection (mainly from 10 to 12 days into the disease) and are useful markers in confirming the diagnosis (Sauerbrei and Wutzler, 2002).

### *1.1.5 HHV-6 and demyelinating diseases*

HHV-6 has characteristics that make it highly capable of causing chronic neurological diseases; it has high prevalence worldwide, latent nature, ability to re-activate, and opportunistic features. It is a highly neurotropic virus and has the capability to penetrate into the CNS, where it stays in a latent form and is able to re-activate if the immune system is compromised (Moore and Wolfson, 2002, Luppi et al., 1994). HHV-6 infection can cause CNS demyelination and loss of astrocytes (Drobyski et al., 1994, Novoa et al., 1997).

HHV-6 was first associated with MS in 1995, when it was found in the postmortem brains of patients that were earlier diagnosed with MS (Challoner et al., 1995). Many studies with brain specimens from autopsies have been carried out since then; HHV-6 infection has been seen in demyelination areas of CNS tissue samples (Carrigan and Knox, 1997). The patients had colonies of HHV-6-infected oligodendrocytes in the white matter of the brain even without histological signs of inflammation or demyelination (Challoner et al., 1995, Friedman et al., 1999). HHV-6 has also been found in postmortem studies in active as well as inactive MS plaques (Cermelli et al., 2003, Virtanen et al., 2005). In addition to postmortem findings, HHV-6 has also been found during the early stages of acute MS plaques in biopsy specimens of the white matter of living patients (Goodman et al., 2003). When autopsy and biopsy specimens are studied, there is a possibility of contamination with body fluids. When this occurs, HHV-6 may accidentally enter the specimen and cause a false positive result.

In most postmortem studies, patients have had an MS diagnosis for a long time and the disease has progressed for many years. As a consequence, the studies give a comprehensive overview of late MS. Studies on serum and CSF samples of patients with early MS give another perspective and an opportunity to compare different disease stages. Serum and CSF samples also enable the diagnosis of ongoing infections. HHV-6 antibodies are often present in early MS (Villoslada et al., 2003). In general, patients with different stages of MS have often HHV-6 DNA in the CSF, especially HHV-6A DNA (Knox et al., 2000, Mancuso et al., 2010, Alvarez-Lafuente et al., 2002, Ablashi et al., 2000, Alvarez-Lafuente et al., 2008, Rotola et al., 2004). In other studies, active viral replication of HHV-6 was discovered during the clinical episodes of relapsing-remitting MS (RRMS) patients (Alvarez-Lafuente et al., 2004, Alvarez-Lafuente et al., 2007). In addition, patients with MS and active HHV-6 replication seemed to have a more aggressive disease course (Alvarez-Lafuente et al., 2006). The

increase of HHV-6 antibodies was connected with RRMS episodes (Simpson et al., 2012, Behzad-Behbahani et al., 2011). An active HHV-6 infection or re-activation is related to MS disease activity in RRMS and secondary progressive MS (SPMS) (Chapenko et al., 2003).

Even though many studies on MS and HHV-6 have been carried out, the association between HHV-6 with demyelinating diseases remains controversial. Several original studies and reviews suggest that there is no relation between MS and HHV-6 infection or re-activation (Hon et al., 2014, Ahram et al., 2009, Swanborg et al., 2002, de Villiers et al., 2006, Taus et al., 2000). Some studies have found no detectable HHV-6 DNA in the CSF or the serum of patients with MS (Gustafsson et al., 2013a, Mirandola et al., 1999). There are also studies and reviews that do not exclude the possibility that a relation between HHV-6 and MS exist, but statistical significance is lacking or there is no clear relation (Voumvourakis et al., 2010, Kuusisto et al., 2008, Ben Fredj et al., 2012, Simpson et al., 2014, Ben-Fredj et al., 2013, Swanborg et al., 2003, Al-Shammari et al., 2003, Enbom et al., 1999). The heterogeneity of the MS disease together with the variability in diagnostic methodology for HHV-6 imposes challenges for the research.

#### *1.1.6 HHV-6 in other neurological diseases*

HHV-6 is known to cause febrile illness and seizures, especially in childhood (Laina et al., 2010). It is capable of causing encephalitis and encephalomyelitis in immunocompromised (Seeley et al., 2007, Baldwin, 2011), as well as immunocompetent patients (Yao et al., 2009, Denes et al., 2004, Isaacson et al., 2005, Pot et al., 2008). Encephalitis may occur with or without the typical skin rash (Yamamoto et al., 2015), and it may have an epileptic onset; also in adults (Merelli et al., 1996).

In some reports, HHV-6 has been linked to chronic fatigue syndrome (CFS) (Ablashi et al., 2000, Komaroff, 2006). HHV-6 antigens have been found in glioma and other brain tumor samples suggesting possible involvement in the pathogenesis of CNS malignancies (Chi et al., 2012, Stodberg et al., 2002). HHV-6 has been reported to cause meningoradiculitis in previously healthy adults (Karam et al., 2009). HHV-6 can cause retrobulbar optic neuritis in HIV-infected patients (Mechai et al., 2007). It has caused optic neuritis to previously healthy adults as well (Moschettini et al., 2006). HHV-6 has also been associated with Mollaret's meningitis and epilepsy (Capouya et al., 2006, Li et al., 2011).

HHV-6 has also been found in the CNS of patients with Alzheimer's disease and with Parkinson's disease (Hemling et al., 2003). On the other hand, it was reported that in both of the diagnosed groups, as well as the reference group, more than three-quarters of the patients had HHV-6 DNA in their brain specimen. This relation was equal in all patient groups, which demonstrates the commonness of HHV-6 in the CNS (Hemling et al., 2003).

#### *1.1.7 Antiviral treatment of HHV-6*

There are no large clinical studies on antiviral treatments of HHV-6 infected adults. Most of the knowledge is based on the results of cell cultures, as well as successful and unsuccessful treatments from small cohorts. Ganciclovir, valganciclovir, cidofovir, and foscarnet are known to be effective antivirals against HHV-6 CNS-infections, and experience is mainly gained from monotherapy and antiviral drug combinations (Table 1).

In cell cultures, foscarnet and ganciclovir are known to inhibit the replication of HHV-6 in human lymphocytes (Akhyani et al., 2006). Foscarnet also effectively inhibits the replication of HHV-6 in neural glial cells (Akhyani et al., 2006).

Acyclovir is not recommended because it has an antiviral effect towards HHV-6 mainly in toxic levels (Manichanh et al., 2000). Valaciclovir has no effect on the presence of HHV-6 DNA in the blood or saliva (Hollsberg et al., 2005). There are successful experiences when treating immunocompetent patients, who have HHV-6 infections with other than CNS disorders, with antivirals (e.g. acute liver failure with valganciclovir or ganciclovir) (Cacheux et al., 2005).

Table 1. Reports of HHV-6 infected adult patients successfully treated with antiviral medication. The main problems (myelosuppression or nephrotoxicity) with the medications are collected from the official instructions made by the pharmaceutical companies and were also discussed in the studies referenced.

<b>Antiviral in clinical use</b>	<b>Trade name in Finland (2015)</b>	<b>Used dose</b>	<b>Immunocompromised patients *</b>	<b>Immunocompetent patients *</b>	<b>The main problems with medication</b>
<b>Ganciclovir</b>	Cymevene <sup>®</sup>	5-10mg /kg/day (i.v.)	Hirabayashi et al., 2013, Zerr et al., 2002, Mookerjee and Vogelsang, 1997, Tokimasa et al., 2002 Ljungman et al., 2007	Denes et al., 2004 Moschetti et al., 2006 Troy et al., 2008	myelosuppression  HHV-6 may have resistance:  Safronetz et al., 2003, Baldwin, 2011
<b>Cidofovir</b>	Cidofovir <sup>®</sup>	375mg /kg/day (i.v.)	Pohlmann et al., 2007		nephrotoxicity
<b>Foscarnet (=phosphonoformic acid)</b>	Foscavir <sup>®</sup>	80-120-180mg /kg/day (i.v.) 4weeks	Vu et al., 2007, Baldwin, 2011, Cole et al., 1998 Pohlmann et al., 2007	Pantry et al., 2013	nephrotoxicity
<b>Valganciclovir</b>	Valcyte <sup>®</sup>	900mg 1x2 (p.o.) 6 weeks		Karam et al., 2009 Isaacson et al., 2005 Pantry et al., 2013 Watt et al., 2012	myelosuppression

\*Reported successful experiences when treating HHV-6 -related CNS disorders of immunosuppressed or immunocompetent patients

### 1.1.8 Radiological features in HHV-6 encephalopathy

In HHV-6 CNS infections, a CT performed during the early phases of HHV-6 infection is usually normal (Noguchi et al., 2010). Typical MRI findings are transient signal intensity abnormalities in the mesial (hippocampus, amygdala) and temporal lobes (Noguchi et al., 2010, Provenzale et al., 2008).

## 1.2 VARICELLA-ZOSTER VIRUS

### 1.2.1 *Introduction and characteristic features of the virus*

VZV is an alpha-herpesvirus that has a high seroprevalence (>95%) in adults in western countries (van Lier et al., 2013, Lee et al., 2013, Salleras et al., 2008). The primary infection usually occurs in childhood between two and six years of age and its clinical manifestation is chicken pox (van Lier et al., 2013). Its typical features are fever with skin vesicles of various stages all over the body (Figures 2 and 3). The incubation period usually ranges from 10 to 21 days. The virus becomes infectious two days before the vesicles appear, and continues to be infectious until the last vesicles have broken down and formed granulation.

VZV also has neurotropic characteristics, and after the primary infection it remains in a latent form in the human host. Latency sites include predominantly the neurons in dorsal root ganglia, cranial nerve ganglia, and autonomic ganglia along entire neuraxis (Gilden et al., 2014, Nagel et al., 2014). VZV can reactivate when the immune system is compromised. A typical clinical feature of re-activation is herpes zoster (shingles). It includes a typical skin rash and pain within 1-3 dermatomes (Figures 4 and 5).

VZV can also cause CNS complications (e.g. meningitis, encephalitis, and acute disseminated encephalomyelitis (ADEM)) that may be fatal (Pahud et al., 2011, Science et al., 2014). CNS-related symptoms may vary from persistent hiccups and vomiting up to generalized seizures (Science et al., 2014, Yoshida et al., 2014).



Figure 2. Chicken pox with a typical rash in a two-year-old child. (Photograph is published with the permission of Lotta Palmén and with the permission of the parents.)



Figure 3. A vesicle in chicken pox (Photograph is authors own material)



Figure 4. Herpes zoster in the thoracic area. (Photograph is authors own material)



Figure 5. Herpes zoster rash. (Photograph is authors own material)



### *1.2.2 Clinical significance in adults*

The VZV primary infection in adulthood is usually more severe when compared to the primary infection that occurs in childhood. In adulthood the disease is more often related with complications and patients might even need hospitalization (Malavige et al., 2008).

VZV may re-activate if the immune system is somehow compromised, but it may cause CNS complications even in immunocompetent adults (Sanguaneko et al., 2015, Pasedag et al., 2014, Nandhagopal et al., 2014, Halling et al., 2014, Klein et al., 2010). Besides herpes zoster and zoster sine herpete, VZV re-activations may cause a variety of neurological and ocular diseases in adults, including meningoradiculitis, cerebellitis, myelopathy, and VZV-vasculopathy (Nagel and Gilden, 2014). Vasculopathy may occur in central or peripheral arteries (Srivastava and Nagpal, 2014).

CNS complications are usually re-activations and they may occur either without a characteristic rash or a delayed rash (Pahud et al., 2011, Sanguaneko et al., 2015). Typical symptoms in VZV CNS infections are headache, fever, and neck stiffness (Becerra et al., 2013). Typical signs of a virus infection have been, however, lacking in approximately half of the cases; patients have lacked typical VZV rash, fever, or even systemic inflammation markers such as CRP and mean leukocyte count (Becerra et al., 2013).

### *1.2.3 Diagnostic tools for diagnosing VZV infection in CNS*

In VZV CNS infections, CSF analyses are the main useful tools in diagnostics. Most adolescents and adults have VZV-serum-antibodies as a sign of an earlier VZV infection, and therefore serum-antibody detection alone is not useful in diagnosis of CNS infections. VZV DNA detection from the CSF is a highly specific and sensitive method in diagnosing VZV CNS infections (Kleinschmidt-DeMasters and Gilden, 2001). In some cases, however, DNA detection may remain negative even if the patient has a clinical VZV CNS infection (Fox et al., 2001). In these cases, a VZV antibody detection from CSF may confirm the diagnosis (Fox et al., 2001). There are a few known reasons why DNA detection or antibody production may give a false negative result in VZV CNS infections. In some cases of VZV CNS infections, some active viral replication may occur in the cerebral arteries, and therefore the virus is not present in the brain parenchyma (Morita et al., 2003). In addition, if the antiviral treatment has been started early, the DNA may not be detectable.

If effective antivirals are started early, they may delay or, in some minority of cases, even prevent antibody production (Linde et al., 1997).

#### *1.2.4 VZV and demyelinating diseases*

The relation between VZV and MS is controversial. Many studies have found some possible association between VZV and MS pathogenesis or the disease course (Ross et al., 1995, Perez-Cesari et al., 2005). Many studies, however, also suggest that no association exists between VZV and MS (Burgoon et al., 2009, Marrie and Wolfson, 2001, Myhr et al., 1998, Lenman and Peters, 1969).

VZV has characteristics that make it potent to cause or trigger MS disease: it is neurotropic, distributed worldwide, and stays in latent form in human host. VZV seems to have increased incidence in both childhood and early adulthood which coincides with MS incidence (Puchhammer-Stockl et al., 2012). VZV seems to be able to cause demyelination in CNS (Hausler et al., 2002). It is also capable of causing recurring episodic neurological infections in immunocompetent patients, which matches the disease course of RRMS (Haug et al., 2010).

It has been reported that patients with a VZV infection have also had signs of demyelination in MRI of the brain (Koskiniemi et al., 2002). A history with VZV infection has been associated with a higher risk for developing MS (Rodriguez-Violante et al., 2009, Ross, 1998). A herpes zoster episode seems to increase the risk of developing MS (Kang et al., 2011). VZV DNA was present in the CSF of patients who were diagnosed with MS, and with most of the patients the DNA was present mainly during their relapses (Sotelo et al., 2008, Ordonez et al., 2010, Mancuso et al., 2007, Sotelo et al., 2014). During MS relapses, VZV DNA was present in the patients' serums as well (Sotelo et al., 2007).

#### *1.2.5 Antiviral treatment and prevention of VZV*

The primary infection of VZV in immunocompetent children usually has a benign disease course and needs no antiviral medication. The most common clinical form of re-activation in immunocompetent adults, herpes zoster, is usually benign as well. It is self-limited, and antiviral medication may not be needed. If the patient is immunocompromised or has some VZV complications, including CNS involvements, antiviral medication is needed (Lionnet et al., 1996). There are several effective antivirals that treat VZV infections: acyclovir, valacyclovir, and famciclovir

(Arvin, 1996). Acyclovir is the safest of the VZV antivirals and is therefore the most used, especially in VZV CNS infections (Lionnet et al., 1996).

There is an effective prevention for VZV infection: live attenuated varicella-zoster virus vaccine. Despite being VZV-vaccinated, patients may have a varicella breakout even with neurological complications (Aslan et al., 2014). It is thought that VZV in a vaccine can induce optic neuritis. The mechanism for vaccine-VZV induced type of optic neuritis is different from the mechanism involved in other post-vaccination optic neuritides (Han et al., 2014). The VZV type used in VZV vaccine may also form a state of latency and, in rare cases, cause clinical re-activation (Tseng et al., 2014). Herpes zoster can be prevented with a vaccine that contains live attenuated varicella-zoster virus. The vaccine was licensed in 2006 and is indicated only for adults. With the vaccination the risk of developing herpes zoster is significantly reduced (Lal et al., 2015).

### 1.3 MULTIPLE SCLEROSIS

#### 1.3.1 *Introduction and clinical characteristics of MS*

Multiple sclerosis is a demyelinating disease that usually affects young adults. Patients are typically previously healthy, more often female than male, and in their thirties.

The incidence of MS has slightly increased in Finland during the last decades and, according to different studies, it varies from 6 to 9 /100000/year (Krokki et al., 2011, Sumelahti et al., 2014). The prevalence in Finland is approximately 103 /100000 and varies notably in different parts of the country (Krokki et al., 2011).

The diagnosis is based on clinical guidelines that have been revised several times, because the clinical diagnostic tools have continuously developed and improved (MRI imaging, laboratory diagnostics). The very first characterization of MS was made in 1868 by Charcot (Landtblom et al., 2010, Poser and Brinar, 2004). In 1931, Allison was the first who divided MS into four different categories and later, in 1954, Allison and Millar together rearranged MS into three categories: early, probable, and possible MS (Poser and Brinar, 2004). The first actual criteria to diagnose clinically-definite MS was created in 1961 and called “the Schumacher criteria” (Poser and Brinar, 2004). In 1980, Bauer was the first who included CSF OCBs in MS criteria and later, in 1983, MRI was also

partly included in the new and more detailed guideline “the Poser criteria” (Poser and Brinar, 2004). Many other revisions and recommendations were also made for each of the characterizations and guidelines. The latest international guideline is based on the criteria of McDonald and was first presented in 2001 and also characterized PPMS (McDonald et al., 2001). The latest generally accepted revision for the McDonald criteria was made in 2010 (Polman et al., 2011).

MS is a disease with a wide variety of different clinical characteristics, and it is described to be more like a syndrome than just a disease (Barnett et al., 2009). There are three common clinical approaches for the different types of disease courses of MS. The different forms of MS are briefly presented below.

The most common form of MS has a disease course with clinical episodes called relapses that are followed with remission stages; relapsing-remitting multiple sclerosis. In Finland, 89% of the MS diagnoses are RRMSs (Sumelahti et al., 2014). The neurological symptoms depend on the location of the inflammatory demyelination in the CNS. Symptoms may be motor or sensory deficiencies, typically unilateral (i.e. weakness, spasticity, paresthesia). The patient may have optic neuritis or diplopia. In some patients, symptoms may include vertigo and problems in controlling the urinary or gastro-intestinal -system. Some patients may also report exceptional fatigue as a symptom. According to the definition, an episode lasts from a minimum of 24 hours to a maximum of 4 weeks (Poser et al., 1983, McDonald et al., 2001). The episode includes either new symptoms from a different CNS region or clear and rapid exacerbation of earlier symptoms. In most cases, symptoms are transient, but some residual symptoms may remain.

RRMS can be diagnosed based on the 2010 revised McDonald criteria (Polman et al., 2011): I) If the patient has had two or more clear distinct episodes (more than 30 days between the episodes) from different CNS regions, at least one of them clinically confirmed, and if the patient has two or more lesions with objective clinical evidence in the MRI, the MS diagnosis is confirmed. II) If the patient has had two episodes, but only one lesion with objective clinical evidence, the dissemination in space should be demonstrated before MS can be diagnosed. III) If the patient has had only one episode and has two or more lesions with objective clinical evidence in MRI, the dissemination in time should be demonstrated before MS can be diagnosed. In Finland, lumbar puncture sampling is still highly recommended in order to distinguish other inflammatory or infectious diseases from MS. Typical CSF finding in MS include the presence of two or more OCBs which highly supports the MS

diagnosis. Also elevated IgG index, that indicates the amount of IgG in CSF compared to amount of IgG in serum, is seen in 70-80% of patients with MS (Andersson et al., 1994).

If a patient has had only one episode and only one lesion with objective clinical evidence, the disease is classified as clinically isolated syndrome (CIS) (Polman et al., 2011). These patients have a higher risk for their disease to progress into MS and in Finland it is recommended to routinely control the clinical neurological condition and perform an MRI 3-12 months after the classification of CIS (based on Finnish Current Care Guideline, updated 3.12.14).

In Finland, 11% of MS diagnoses are classified as primary progressive (PPMS) types (Sumelahti et al., 2014). Patients with PPMS do not typically have distinguishable episodes, but the disease and the disability of the patient progress continuously. PPMS can be diagnosed based on the McDonald criteria revised in 2010 (Polman et al., 2011): If the patient has had a disease progression for a year or longer and has two of the following criteria: evidence for the dissemination in space either I) in the brain or II) in the spinal cord or III) a positive CSF finding with an elevated IgG index or OCBs.

When the daily disability of the patient in RRMS type of disease course starts to worsen between relapses or without relapses, the disease has progressed to a secondary progressive multiple sclerosis (SPMS).

If the patient has typical MS lesions in MRI but no clinical symptoms of CIS or MS, the condition is called "radiologically isolated syndrome" (RIS). It is usually a coincidental finding, when the patient has undergone MRI imaging for another reason than a suspicion of demyelination. Approximately 30% of the patients with RIS seem to have neurological episodes and some of these progress into CIS or MS within five years (Granberg et al., 2013, Okuda et al., 2014). In Finland, patients with RIS are not recommended to have specific routine controls.

### *1.3.2 Pathogenesis of MS*

Environmental factors, genetic predisposition, and infective factors, similar to other autoimmune diseases, play a role in MS pathogenesis (Karussis, 2014). The main pathological event is the destruction of myelin in the CNS, but also neurons are damaged depending on the stage of the disease course. During the early stages of MS oligodendrocytes and myelin sheaths in the lesions are destroyed but axons and neurons remain mainly uninjured (Lucchinetti et al., 2011). The MS

plaque is not in a static state (Wu and Alvarez, 2011). A single active MS plaque in the CNS contains complex morphological features that depend on disease duration (Barnett et al., 2009). The disease mechanisms also differ among the natural disease courses of MS (Lassmann, 2013). Even the target antigens seem to vary in the disease progression (Sellebjerg et al., 2000).

#### 1.3.2.1 Cell-mediated autoimmunity theory of MS

Different T cells are thought to be the main agents in the cell-mediated autoimmune reaction in MS pathogenesis. In particular, CD4<sup>+</sup> T cells have a role in both cell-mediated and in humoral autoimmune reactions. These CD4<sup>+</sup> T cells may incorrectly identify an auto-antigen and lead to an attack against the host's own myelin in the CNS and cause demyelination. A few auto-antigens are known, which these T cells recognize: myelin basic protein (MBP), myelin oligodendrocyte glycoprotein (MOG), myelin-associated glycoprotein, and proteolipid protein (PLP). These auto-antigenic T cells are found in patients with MS and in reference controls as well, and it is thought that in MS disease some agent incorrectly activates these T cells (Hellings et al., 2001). Autoreactive CD8<sup>+</sup> T cells are also found in both patients with MS and reference controls, but they may differ in targets that activate them (Berthelot et al., 2008). Regulatory T cells (Treg) control host auto-antigenic T cells and their defective function is seen in patients with MS (Costantino et al., 2008). In addition, apoptosis of incorrectly-activated auto-reactive T cells is not activated properly or is actively inhibited in patients with MS, which is thought to be another mechanism for demyelination in the CNS (Moreno et al., 2014, Sharief, 2000, Saresella et al., 2005, Sharief and Semra, 2001).

#### 1.3.2.2 Humoral autoimmunity theory of MS

The cells in the humoral immune system are also thought to be associated with MS pathogenesis. Naïve CD4<sup>+</sup> cells are activated by dendritic cells (DCs) that serve as antigen-presenting cells in the immune system. In MS, these DCs occur in activated phenotypes (Grigoriadis and van Pesch, 2015, Xie et al., 2015). CD4<sup>+</sup> T-helper cells can also incorrectly recognize auto-antigen, either spontaneously or by activation of auto-antigen-specific B cells (Harp et al., 2010). The activated T-helper cell then incorrectly activates other B cells to attack against the host's CNS. CNS-specific B-cells have been found from patients with MS (Kuerten et al., 2014).

B cells migrate from peripheral blood into the CNS through the blood-brain-barrier (BBB) and mature in the CNS into plasma cells- /clonally-expanded plasma cells that produce IgG antibodies

seen as OCBs (Bankoti et al., 2014, von Budingen et al., 2010). MS lesions with clear demyelination and astrogliosis contain high amounts of IgG (Glynn et al., 1982). Antibodies signal macrophages to incorrectly cause host myelin destruction. Active MS plaques are known to contain myelin-laden or foamy macrophages, indicating ongoing demyelination (Lucchinetti et al., 2011).

B cells seem to have a strong role in the immunopathogenesis of MS. Besides the ability of B cells to activate T cells, their presence affects proliferation of the T cells (reduces when are not present) (Bar-Or et al., 2010). Virus-specific B cells are also capable of becoming active without the help of T cells (Hebeis et al., 2004).

#### 1.3.2.3 Viral theories of MS pathogenesis

During the past decades, numerous different viruses have been proposed to be involved in MS pathogenesis. In the 1970s, the measles was strongly associated with MS (Salmi et al., 1972). Other candidates included HSV (Fraser et al., 1972) and an MS-associated agent, also called “Carp agent” (Carp et al., 1972, Koldovsky et al., 1975). In the 1980s, on the other hand, EBV was associated with MS and still is the strongest candidate to date (Belbasis et al., 2015). A new retrovirus was also found at this time, which was soon connected with MS as well (Greenberg et al., 1989, Perron et al., 1997).

Different viruses are thought to be able to cause demyelination in both the cell-mediated and humoral immune pathways. Viruses may activate B cells to produce cross-reacting antibodies or they may activate T cells (with molecular mimicry) to destroy host oligodendrocytes (Venkatesan and Johnson, 2014). For example, EBV-reactive CD8<sup>+</sup> T cells are intrathecally enriched in some of the patients with MS and these cells also show increased cytolytic activity against EBV (Lossius et al., 2014). The death of oligodendrocytes, that eventually leads to CNS demyelination, may also occur via direct virus infection in oligodendrocytes that induces lysis or apoptosis of the cells (Venkatesan and Johnson, 2014).

#### 1.3.3 Treatment of MS

There is no curative medication to treat MS so far. It is, however, possible to slow down the disease progression and reduce the relapses with anti-inflammatory drugs. Most of the new and effective drugs against MS relapses have an impact on leucocyte function and reduce their number. Different drugs against RRMS are briefly presented in Table 2.

Table 2. Immunotherapies used in RRMS treatment in Finland (based on Current Care Guidelines in Finland, Käypä hoito, updated 3.12.2014)

Immunomodulator	Target	Mechanism of action	Reference
Beta-interferon 1a, 1b	Binds to type-1 IFN receptor on the cell surface	Prevents lymphocyte traffic through blood-brain barrier, inhibits T cell function, modifies the differentiation of T cells	Dhib-Jalbut and Marks, 2010, Kalinke and Prinz, 2012
Glatirameracetate	Synthetic polypeptide with similarity to myelin basic protein	Prevents the action of T cells against myelin basic protein	Racke and Lovett-Racke, 2011
Teriflunomid	Dihydro-orotate dehydrogenase inhibitor	Prevents the proliferation of lymphocytes	Bar-Or et al., 2014
Dimethyl fumarate	The nuclear factor (NrF2) transcriptional pathway activator	Enhances anti-inflammatory effect and reduces the effects of oxidative stress	Gold et al., 2012, Viglietta et al., 2015
Natalizumab	Humanized anti- $\alpha$ 4-integrin monoclonal antibody	Prevents lymphocyte traffic into the CNS	Krumbholz et al., 2012
Fingolimod	Sphingosine 1-phosphate receptor modulator	Prevents lymphocyte traffic from the lymph nodes	Cohen et al., 2010
Alemtuzumab	Humanized anti-CD52 monoclonal antibody	Decreases number of CD52-expressing cells (some of the B and T cells and monocytes) from the circulation	Freedman et al., 2013



## 1.4 OLIGOCLONAL BANDS

### 1.4.1 Introduction of OCBs

The most important OCBs are IgG class antibodies that are produced in the CSF by clonally expanded plasma cells (von Budingen et al., 2010). Isoelectric focusing (IEF) is well established in clinical use for detecting OCBs. The main benefit of IEF is its higher sensitivity, when compared to other methods (Mygland et al., 2007). If a patient has two or more visible bands in the CSF, the OCB result is defined to be positive (Figure 6). True IgG-OCBs that are counted as bands in patients with MS, are visible only in CSF and they do not have visible counterparts in the serum (Davenport and Keren, 1988).

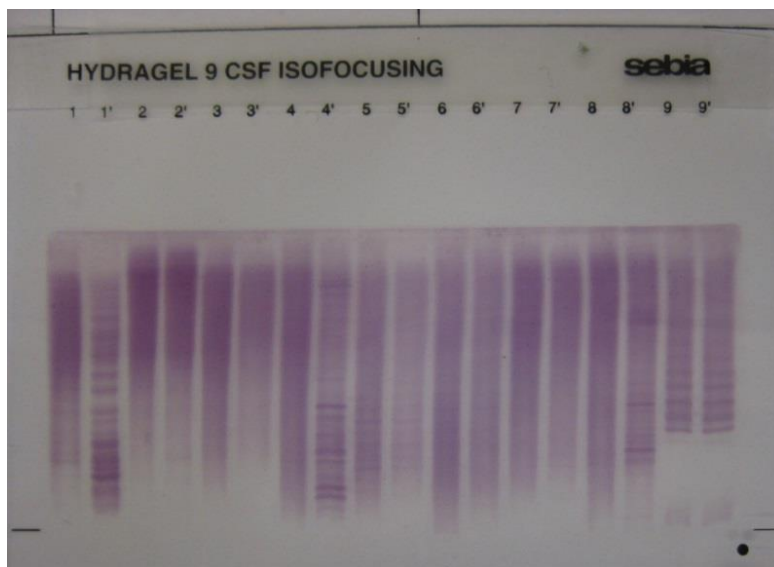


Figure 6. IEF gel after an IEF run and immunofixation. Serum (n) and CSF (n') samples are compared and clear bands that are seen in CSF and not in serum are defined as positive OCB findings (as seen in samples: 1, 4, and 8) Samples 9 and 9' contain a positive control. (Photograph is authors own material)

#### *1.4.2 OCBs in clinical use*

More than 95% of the patients with MS have OCBs in their CSF (Andersson et al., 1994). OCBs are not specific to MS and they can also be seen in other neurological conditions. Even though OCB status is no longer required in MS diagnosis, the presence of OCBs remains an important part for an MS diagnosis and OCB positivity has predictive value for CIS patients' disease progressing into MS (Dobson et al., 2013, Paolino et al., 1996, Zipoli et al., 2009). It has even been recommended that if a patient with MS has no OCB in the CSF, it is in most cases a possible sign of a misdiagnosis of MS (Dobson et al., 2013, Zeman et al., 1996).

#### *1.4.3 OCBs in neurological diseases*

The presence of CSF-specific OCBs is related to immunological reactions in the CNS. Besides MS, OCBs may also be present in some patients with other different autoimmune diseases with neurological involvements (e.g. systemic lupus erythematosus, Sjögren's syndrome, neurosarcoidosis) and also in some patients with neurodegenerative diseases (e.g. Alzheimer's disease and other dementias) (Petzold, 2013, McLean et al., 1995, Janssen et al., 2004). Few OCBs have been found in patients with postencephalitic Parkinsonism (Williams et al., 1979). OCB positivity has even been observed in some patients who have had a stroke (Pruss et al., 2012). It is thought that some immunological exposure may be related to the pathogenesis of these kinds of strokes. OCBs have also been observed in structural central nervous system lesions and in spinal arteriovenous malformations with no signs of any CNS infections (Cohen et al., 2000).

The prevalence of OCBs in the CSF of patients with MS differs worldwide (e.g. in China only 63% of patients had a positive OCB result), and it is thought to be dependent on different immunogenetic backgrounds (Li et al., 2007). In different countries the OCB status seems to give a different prognosis; in a Turkish study, OCB positivity, unlike in other studies, was associated with a better prognosis with better result in Expanded Disability Status Scale (EDSS, scale that quantifies disability in multiple sclerosis), better clinical course with lower relapse severity (Idiman et al., 2009). In another study, OCB status did not seem to be associated with the clinical progress in MS patients (Lourenco et al., 2013). OCB positivity is also associated with increased white matter lesions in MS patients, as well as greater global brain atrophy and increased CSF and ventricular volume (Ferreira

et al., 2014). In addition, it has been reported that no relation has been found between the OCB status and the number of lesions or the pathology in the CNS (Ellidag et al., 2013).

It is known that, in rare cases, some MS patients truly lack OCBs. These patients are thought to have more of a benign disease course (Zeman et al., 1996, Lechner-Scott et al., 2012, Joseph et al., 2009, Sa et al., 2005). OCB-negative patients seemed to have a different immunogenetic background and they may form a subgroup within MS disease (Fukazawa et al., 1998).

OCBs are also seen in different neurological infections, where they are often produced against the infectious agent (e.g. measles in subacute sclerosing panencephalitis) (Vandvik and Norrby, 1973) (Table 3).

#### 1.4.4 OCBs reactivity against different pathogens

OCB reactivity has been found not only against different CNS virus infections, but also against other viruses and Chlamydia pneumoniae, also in MS (table 3).

Table 3. OCBs reactivity against different pathogens in different diseases

Pathogen	Disease	References
<b>VZV</b>	Vasculopathy	Burgoon et al., 2003, Luxton et al., 1995, Vartdal and Vandvik, 1983
<b>EBV</b>	MS	Franciotta et al., 2011, Virtanen et al., 2014, Rand et al., 2000, Castellazzi et al., 2014
<b>Measles</b>	Subacute sclerosing panencephalitis	Vandvik and Norrby, 1973, Mehta et al., 1982, Luxton et al., 1995, Vartdal and Vandvik, 1983
<b>Chlamydia pneumoniae</b>	MS	Franciotta et al., 2005, Yao et al., 2001
<b>HSV</b>	Encephalitis	Luxton et al., 1995

## **2 AIMS OF THE STUDY**

The general objective of this doctoral study was to investigate the possible role of viruses in MS pathogenesis. The aim was to focus on HHV-6A, HHV-6B, and VZV -antibodies and their possible presence during the early stages of MS disease.

The specific aims in each study were:

- To study clinical data of patients who have had a serologically confirmed acute HHV-6 infection and investigate their possible signs of MS. (I)
- To study if OCBs in the CSF contain antibodies that react with HHV-6A or HHV-6B antigens in patients with early stage MS or other demyelinating diseases. (II)
- To study if OCBs in the CSF contain antibodies that react with VZV antigens in patients with early stage MS or other demyelinating diseases. (V)
- To study if patients, who have HHV-6A, HHV-6B or VZV -reactive OCBs in their CSF, have some identifiable features including clinical differences, or difference in laboratory or MRI findings. (III-V)

### 3 PATIENTS AND METHODS

#### 3.1 PATIENTS

##### 3.1.1 *Patients (I-V)*

(I) Patients were included in this study from an earlier study of serological survey of patients with MS. The study design and procedure of patient collection are described in the original study (Virtanen et al., 2007a). All patients who had serologically confirmed acute or chronic HHV-6A infection (N=9) were selected for detailed evaluation of their clinical data. Eventually twelve patients underwent retrospective evaluation of previous clinical data of their neurological history. Three patients with other neurological diseases were controls.

(II-V) All the patients who had OCBs in their CSF and were referred by general practitioners for a neurological evaluation, to the Department of Neurology at the University Hospital of Helsinki, had serum and CSF samples saved for further retrospective analysis. Systematic collection of all the paired serum and CSF samples lasted 365 days. During the collection period, altogether 109 patients had a positive result for CSF OCB detection. Samples of 30 patients with positive results in OCB detection were missing and not available for further analysis. All the samplings were clinically indicated because patients had symptoms that led to a suspicion of an inflammatory disease of the CNS. Every patient who had a suspicion of demyelinating or inflammatory disease (at the Department of Neurology, University Hospital of Helsinki) underwent lumbar puncture sampling, according to the protocol of the clinic. Every patient diagnosed with MS was treated based on the Current Care Guidelines used in Finland.

##### 3.1.2 *Samples (I-V)*

From all our studied patients, both serum and the CSF samples were collected at the same time at the beginning of the follow-up. None of the patients had corticosteroid treatment prior to lumbar puncture sampling. None of the patients had any immunotherapy (Table 2) before the lumbar puncture. Routine clinical tests of CSF (IgG index, protein concentration, white blood cells (WBC) and red blood cells (RBC) counting, glucose level, albumin level)

were analyzed soon after sampling. Samples were stored at -70 degrees before further HHV-6 or VZV analysis.

### 3.1.3 *Ethical approval*

The Ethics committee of Helsinki University Central Hospital has approved all the studies (I-V).  
Dnro 644/E9/02

## 3.2 METHODS

### 3.2.1 *Antibody tests of HHV-6A and HHV-6B (I-IV)*

Immunofluorescence assay (IFA) was used to analyse the level of antibodies in the serum. Specific antigens used in IFA analysis were HHV-6A strain GS that was grown in HSB-2 T cell line and HHV-6B strain Z29 that was grown in MOLT-3 T-cell line. Serum samples were diluted and organized in reciprocal series. The mildest dilution that gave clear green fluorescence light above negative control was determined to be the titer. (Virtanen et al., 2007b)

### 3.2.2 *Avidity test (I-IV)*

Avidity testing was used to determine whether the antibodies of the patient are residues from a past infection or from a primary infection, i.e. an ongoing or recent initial infection. Avidity tests of IgG antibodies binding capacity to antigen were tested by using urea wash (Virtanen et al., 2007b). IgG antibodies with high binding capacity indicate serological past infection and antibodies with low binding capacity indicate serological primary infection.

### 3.2.3 *OCB detection (II-V)*

Paired serum and CSF samples were diluted to the same IgG concentration (usually 20 mg/l). The amount of CSF and diluted serum used in this method was 10 µl. IgG antibodies were separated on agarose gel by isoelectric focusing (IEF) (pH gradient 3.5-9.5) using Sebia

Hydragel 9 CSF isofocusing system on the Sebia Hydrasys Focusing apparatus (Sebia, Lisses, France). IgG oligoclonal bands were localized in the CSF by performing immunofixation with immunoperoxidase staining. Two or more visible bands in CSF with no serum counterparts were determined to be an OCB-positive result. Positive and negative controls were included in every series.

#### *3.2.4 HHV-6 and VZV-reactivity in OCBs (II-V)*

Affinity driven immunoblot was used to localize HHV-6 and VZV -reactive OCBs. Nitrocellulose membrane was coated with HHV-6A (Advanced Biotechnologies, Columbia, MD) or 6B (Meridian Life Science, Saco, ME) or VZV viral antigens. The VZV antigens were provided by the Department of Virology of HUSLAB as a concentrated solution of extracted antigens produced by expression in A549 cells. Paired CSF and serum samples of the patients were separated by IEF on agarose gel (see “OCB detection” above). The gel was then in direct contact overlaid with the nitrocellulose membrane coated with the viral antigen. Passive transfer of proteins was performed under pressure of one kilogram for 30 min. Transferred IgG bands were then detected with alkaline-phosphatase conjugated anti-human IgG (1:1000, DAKO, Glostrup, Denmark) and visualized using 4-nitro blue tetrazolium chloride (NBT) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP). Positive and negative controls were used in every series. Two or more virus-reactive bands in CSF with no serum counterparts were determined to be an OCB-positive result.

#### *3.2.5 Clinical data (I-V)*

In the clinical evaluation, earlier neurological history of the patients was collected retrospectively using a systematic form (in supplements). The clinical evaluation also included prior medications and treatments of the patients. Clinical data of neurological history contained all the documents that were available for the neurologists who were responsible for the examination, diagnosis, and treatment of the patients. Clinical re-evaluation was repeated after follow-up time ended.

(III-V) Follow-up time lasted a minimum of two years and was started after the end of the systematic collection of the samples and neurological diagnoses were re-evaluated according to McDonald's criteria (See 1.3.1 *Introduction and clinical characteristics of MS*).

### 3.2.6 *Neurological diagnoses (I-V)*

All the neurological diagnoses were confirmed by a neurologist. All diagnoses were made blindly without virus-reactive OCB results. All MS diagnoses were based on current guidelines of that time. All the patients whose disease characters fulfilled McDonald's diagnostic criteria were classified as MS. If patients had their first clinically-confirmed demyelinating event and their MRI of the CNS didn't fulfil Barkhof's criteria (Barkhof et al., 1997), the disease was classified as clinically isolated syndrome (CIS). All other neurological diagnoses were classified to be "other neurological diseases" (OND).

### 3.2.7 *Statistical analysis (III, V, thesis)*

We used non-parametric Mann-Whitney U test in comparing differences between groups. We used PASW Statistics 18 software for calculating statistical tests. All our tests were two-tailed. P-values less than 0.05 were considered statistically significant.



## 4 RESULTS

### 4.1 HHV-6 SEROLOGICAL PRIMARY INFECTION AND MS (I, III, IV)

Of the 23 patients with, serologically-confirmed primary HHV-6A or HHV-6B infection, we found 18 who had serologically confirmed primary infection during or near the clinically-distinct demyelinating event (Table 4). Of those 18 patients, 11 were diagnosed with MS soon after the clinical and laboratory investigations (Table 6). None of the patients who had a primary HHV-6 infection had any typical signs of a virus infection (e.g. fever, rash).

Patients who had serological primary HHV-6 infections were from 18 to 53 years old (mean 35 years). Of the patients, 13 were female (57%) and 10 were male (43%).

Many patients had clear time-dependent relation between HHV-6A/B serological primary infections and their clinically-confirmed neurological symptoms of demyelination (Figure 7). There were patients with only HHV-6A- (43%, n=10) or only HHV-6B-primary infections (35%, n=8). Five of the patients had serological primary infection with both HHV-6A and HHV-6B at the same time (22%, n=5).

Primary HHV-6 infections were also seen in some other neurological diseases, besides MS or CIS: one patient was diagnosed with Mollaret's meningitis and one patient had epilepsy. More detailed presentation of patients is in Table 4.

HHV-6 analysis was made retrospectively for all of the patients and therefore no one received antiviral treatment in spite of the serological signs of an acute infection.

Table 4.

Patient number	Serological primary infection	Diagnosis at the beginning of follow-up time	Diagnosis after the 2-years follow-up time	Age	F / M	Time in months between latest neurological symptoms to sampling
1	6A	ADEM	ADEM	47	F	1
2	6A	MS	MS	33	M	2
3	6A	epilepsy	epilepsy	21	F	-
4	6A	MS	MS	48	F	2
5	6A	PPMS	PPMS	38	M	0
6	6A	MS	no follow-up	35	F	3
7	6A	MS	no follow-up	27	F	6
8	6A	CIS	no follow-up	49	F	3
9	6A	CIS	no follow-up	39	F	2
10	6A	CIS	no follow-up	36	M	years *
11	6B	tension neck	no follow-up	23	M	-
12	6B	depression	no follow-up	39	M	-
13	6B	MS	MS	39	F	0
14	6B	CIS	Mb n.peronei and	48	M	0
15	6B	MS	MS	31	F	3
16	6B	CIS/RIS	CIS	53	F	4
17	6B	CIS	CIS	29	M	0
18	6B	MS	MS	48	M	years *
19	6A+6B	MS	MS	21	F	4
20	6A+6B	MS	MS	40	M	0
21	6A+6B	Mollaret's meningitis	Mollaret's meningitis	18	M	-
22	6A+6B	MS	MS	44	F	0
23	6A+6B	RA	MS	47	F	2

F/M

female / male

MS

multiple sclerosis, relapsing-remitting disease course

CIS

clinically isolated syndrome

ADEM

acute disseminated encephalomyelitis

RA

rheumatoid arthritis

PPMS

primary progressive MS

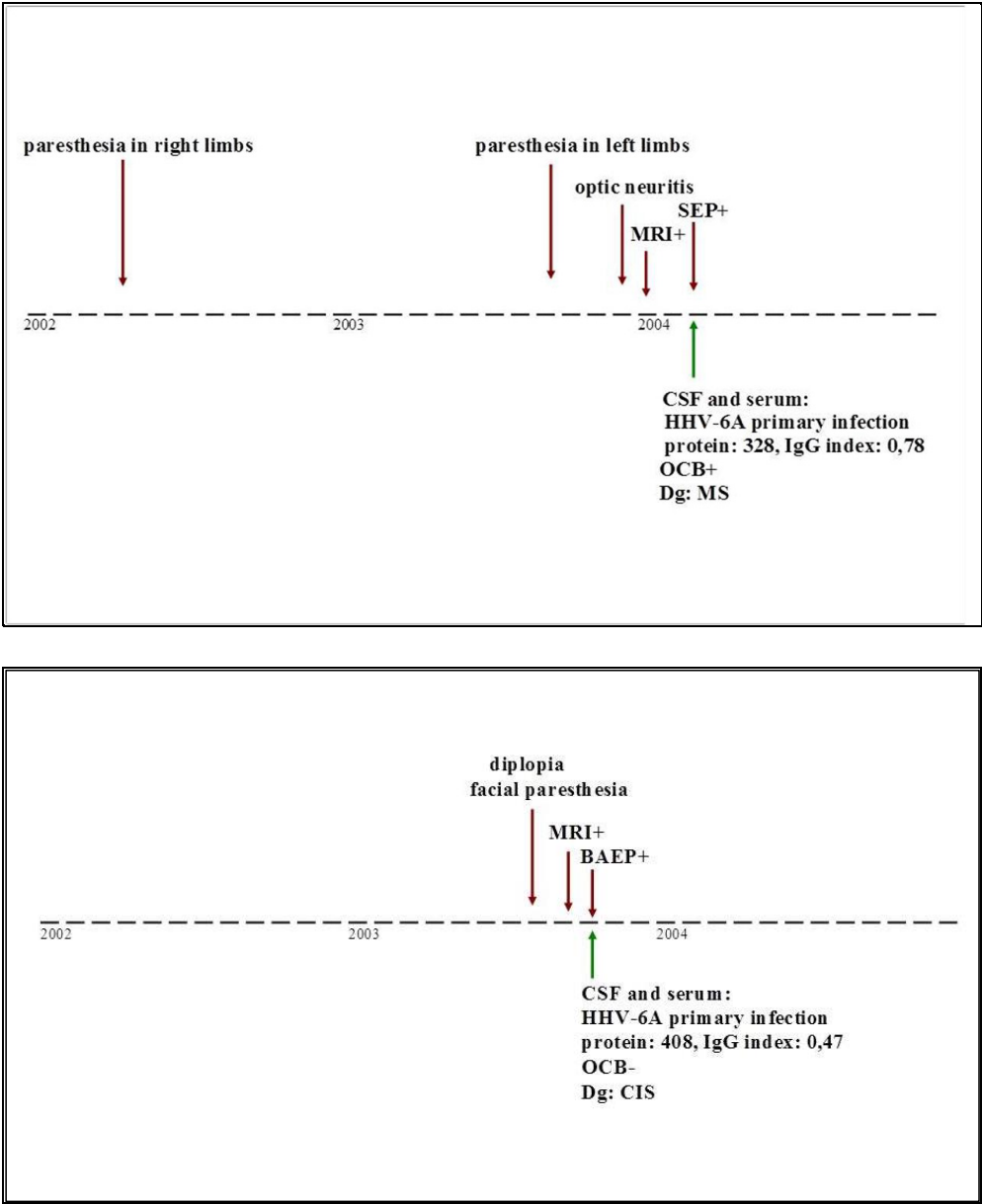
RIS

radiologically isolated syndrome, patient had no clinical episode

\*

atypical symptoms during the lumbar puncture

Figure 7.     Diagrams of two patients with primary HHV-6A infections and time-dependency of its relation with demyelinating events. One was soon diagnosed with MS and the disease of the other one was classified as CIS. (SEP= somatosensory evoked potential, BAEP= brainstem auditory evoked potential, protein= protein concentration in CSF)



## 4.2 VIRUS-REACTIVE OCBs (II-V)

Of 79 patients, altogether 26 (33%) had HHV-6A and/or HHV-6B and/or VZV –reactive OCBs in their CSF. Of 17 patients who had HHV-6-reactive OCBs, altogether 35% (n=6) had both HHV-6A and HHV-6B-reactive bands, 18% (n=3) had only HHV-6A-reactive OCBs, and 30% (n=5) had only HHV-6B OCBs. Of 12 patients who had VZV-reactive OCBs, 3 had concurrent HHV-6 –reactive OCBs (Table 5).

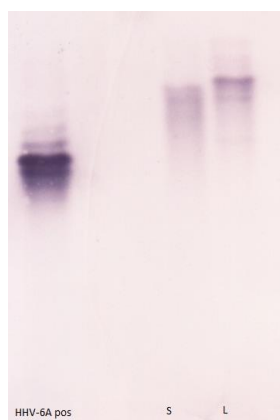


Figure 8. Nitrocellulose membrane with HHV-6A –reactive OCBs. In “HHV-6A pos” contains positive control, two columns of negative controls are un-named in the photograph, “S” contains serum of the patient, and “L” contains CSF of the patient. In CSF (L) there are visible bands that don’t have serum counterparts (S). (Photograph is authors own material)

Virus-reactive OCBs were present in patients CSF near the event of their clinical neurological symptoms that led to a suspicion of demyelinating disease (Table 5). Overall 62% (n=16) of the patients were diagnosed with MS soon after the lumbar puncture. The disease of seven patients was classified as CIS.

No significant differences were evident in the clinical courses or symptoms among patients who had virus-reactive OCBs in their CSF and patients who had no virus-reactive OCBs. Most of the patients with MS, who had virus-reactive OCBs, had relapsing-remitting disease course and only one patient had PPMS. During the follow-up time of two years, the diagnosis of one patient progressed from CIS to MS, and the diagnosis of one patient was re-classified from MS to CIS. (Table 5)

Every patient was treated according to the Current Care Guidelines used in Finland.

Table 5. All patients who had virus-reactive OCBs

<i><b>Patient</b></i>	<i><b>Virus-reactive OCB</b></i>	<i><b>Female (F) / male (M)</b></i>	<i><b>Age</b></i>	<i><b>MRI (Barkhof x/4)</b></i>	<i><b>Dg after lumbar puncture, at the beginning of follow-up</b></i>	<i><b>Dg after 2 years follow-up</b></i>	<i><b>Number of clinical episodes after 2 years follow-up</b></i>
1	HHV-6A+6B	F	48	4	MS	MS	3
2	HHV-6B	F	30	0	MS	MS	4
3	HHV-6A+6B	M	32	3	CIS	CIS	
4	HHV-6A	F	29	3	MS	CIS	
5	HHV-6A	F	47	4	ADEM	ADEM	
6	HHV-6A	M	39	4	MS	MS	2
7	HHV-6A+6B	F	29	3	MS	MS	3
8	HHV-6A+6B	F	45	0	paresthesia	non-specific neurological symptoms	
9	HHV-6A+6B	M	31	4	MS	MS	1
10	HHV-6B	F	29	0	CIS	CIS	
11	HHV-6B	F	21	0	MS	MS	3
12	HHV-6B	M	40	4	MS	MS	4
13	HHV-6A+6B	F	21	3	PPMS	PPMS	
14	HHV-6B	F	33	1	CIS	CIS	
15	VZV	M	31	3	CIS	CIS	
16	VZV	F	49	0	Mollaret's		
17	VZV	F	25	3	MS	MS	2
18	VZV	F	37	1	CIS	CIS	
19	VZV	F	32	4	MS	MS	1
20	VZV	M	44	3	CIS	CIS	
21	VZV	M	31	4	MS	MS	1
22	VZV	F	39	0	CIS	MS	1
23	VZV	F	35	2	MS	MS	4
24	HHV-6A+VZV	F	29	3	MS	MS	2
25	HHV-6A+6B+VZV	F	25	4	MS	MS	3
26	HHV-6A+6B+VZV	F	16	3	MS	MS	3

Patients with HHV-6 or VZV-reactive OCBs had significantly higher IgG index ( $p=0.007$ ) and more OCBs ( $p=0.003$ ) in their CSF in comparison to patients with no virus-reactive OCBs in their CSF (Table 6). Patients with virus-reactive OCBs also had lower protein concentration and they were also younger than the patients with no virus-reactive OCBs, but these findings were not statistically significant.

Table 6. Patients with CIS or MS

	Virus-reactive OCB	No virus- reactive OCB	All patients	Significance of difference, p-value*
All / M / F (n)	23 / 7 / 16	42 / 13 / 29	65 / 20 / 45	NS
Age (mean)	31 (SD 7.5)	35 (SD 9.1)	34 (SD 8.7)	NS
IgG index <sup>1</sup> (mean)	1.37 (SD 0.63)	1.02 (SD 0.50)	1.15 (0.57)	0.007
MRI, Barkhof x/4	2.5 (SD 1.5)	2.9 (1.3)	2.7 (SD 1.3)	NS
Protein concentration <sup>2</sup> (mean)	341	376	363	NS
WBC count <sup>3</sup> (mean)	10	10	10	NS
No. of all OCBs (mean)	21 (SD 5.4)	16 (SD 6.7)	17 (SD 6.6)	0.003

\*Significance of difference when comparing patients with HHV-6 or VZV-reactive OCBs in their CSF with patients who have no virus-reactive OCBs. P-values less than 0.05 were considered statistically significant.

Reference values:

<sup>1</sup> 0.34-0.60

<sup>2</sup> 150-450 mg/l

<sup>3</sup> 0-5 x 10<sup>6</sup>/l

Of the patients with virus-reactive OCBs, 8 had both HHV-6A and HHV-6B –reactive OCBs in their CSF. Three patients had VZV and HHV-6 –reactive OCBs in their CSF in early MS (Figure 9).

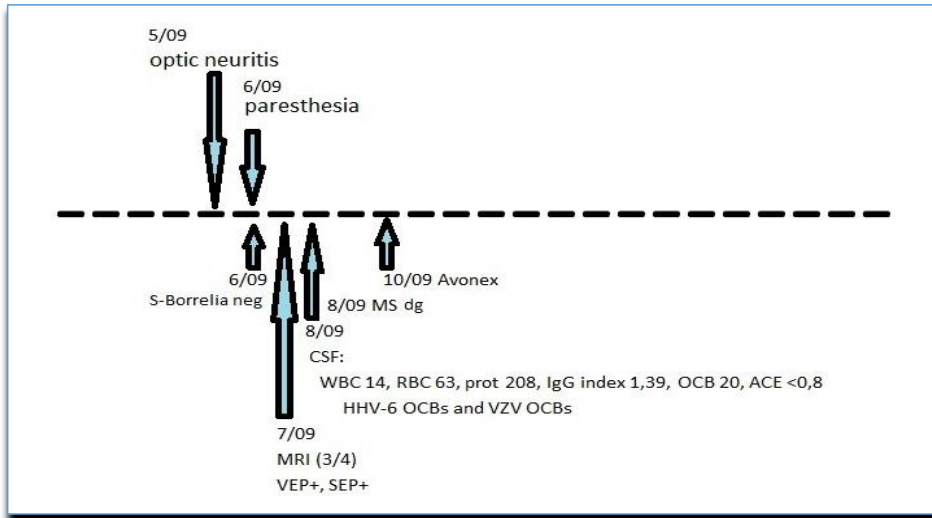


Figure 9. Patient is a 16-year-old previously-healthy female, who had both HHV-6 and VZV- reactive OCBs in her CSF in early MS. (Avonex= Beetainterferon 1a, ACE= Angiotensin-1-converting enzyme, prot= protein concentration, S-Borrelia= serum antibodies of Borrelia Burgdorferi, VEP= visual evoked potential, SEP= somatosensory evoked potential).

We collected differences in several parameters from separate studies (III, V, thesis): between patients who had HHV-6 reactive OCBs and who had no HHV-6 reactive OCBs, between patients who had VZV-reactive OCBs and who had no VZV-reactive OCBs, and between patients who had HHV-6A and / or HHV-6B and /or VZV –reactive OCB and patients who had no virus-reactive OCBs at all. Differences were seen in patient age, their number of total OCBs in CSF, amount of protein concentration in CSF, and level of IgG index. In separate studies, differences in different parameters were parallel. The significant differences were in the different parameters. (Table 7)

Table 7. Statistical comparison of the differences in parameters of patients who had HHV-6 or VZV –reactive OCBs in their CSF comparing patients who had no HHV-6 or VZV –reactive OCBs. Differences are collected from different studies to the same table. Significant p-values ( $p < 0.05$ ) are presented; non-significant (NS).

	<i>Patients with HHV-6-reactive OCBs compared to patients with no virus-reactive OCBs</i>	<i>Patients with VZV-reactive OCBs compared to patients with no virus-reactive OCBs</i>	<i>Patients with both HHV-6-reactive and/or VZV-reactive OCBs compared to patients with no virus-reactive OCBs</i>
<i>Younger age</i>	0.047	NS	NS
<i>More OCBs in CSF</i>	0.001	NS	0.003
<i>Lower protein concentration in CSF</i>	NS	0.012	NS
<i>Higher IgG index</i>	NS	0.014	0.007



## 5 DISCUSSION

### 5.1 SEROLOGICALLY CONFIRMED HHV-6 IN MS

Several patients were found with a serological primary infection as a sign of an active HHV-6 infection in early MS. This agrees with earlier studies, where some of the patients with MS seem to have a systemic HHV-6 infection or activation seen as a presence of active antibodies or HHV-6 DNA in the serum (Akhyani et al., 2000, Caselli et al., 2002, Berti et al., 2002, Friedman et al., 1999, Tomsone et al., 2001). Systemic activation of HHV-6 or infection by HHV-6 in MS is controversial and in some studies there are no signs of an active HHV-6 infection in peripheral blood (PB) (Rotola et al., 1999).

In comparison to HHV-6B, HHV-6A is more often associated with MS and other chronic neurological diseases, but surprisingly many patients carried HHV-6B-primary infections and some seemed to have serological signs of a co-infection. Usually, HHV-6A is known to cause a symptomless primary infection shortly after HHV-6B primary infection in childhood (Agut, 2011). It is possible that the same pattern of co-infection may also be seen in adulthood.

One of the weaknesses in our serum-sample study is that there were no previous serum samples from the patients' childhood nor from the time before their neurological symptoms. Such samples would have reliably shown if our patients with serologically confirmed primary HHV-6A or HHV-6B infections truly lacked HHV-6 antibodies before their neurological symptoms and if this HHV-6 infection was their first HHV-6 infection. We have no systematic reliable data on the childhood infections of the patients because the clinical evaluation was based on a retrospective collection of clinical neurological data and the patients had no interviews nor questionnaires about their HHV-6 infection history. The seroprevalence of HHV-6 is not 100% in adulthood and, therefore, it is possible that the serologically confirmed primary HHV-6 infections may be true and not re-activations.

Even though HHV-6A and HHV-6B are separate viruses, they have been analyzed and discussed together in some parts of the study. In both serological and CSF studies, almost half of the HHV-6 cases were associated with HHV-6A and the rest with HHV-6B. The overall number of HHV-6 cases in our studies was too low for further relevant comparison between these viruses, and therefore we didn't separate them. In our study, however, the HHV-6 findings were clearly not associated with only one of the viruses.

None of the patients had typical signs of an active virus infection (i.e. fever, skin rash). HHV-6 may occur without any typical skin rash (Sloots et al., 1995, Yoshikawa et al., 2001). It seems that HHV-6 may manifest without the typical symptoms of viral CNS infection in immune-competent adults, in contrary to immunocompromised patients (Al Fawaz et al., 2014, Astriti et al., 2006).

In many studies, the focus has been on the detection of viral DNA. It is known that low viral loads are often seen in acute CNS infections of herpesviruses (Gaeta et al., 2009). In addition, postmortem studies of patients with HHV-6 infections have shown that in some cases a low amount of HHV-6 DNA is seen in the CSF and a high load of HHV-6 remains in the CNS tissue (Fotheringham et al., 2007). It is therefore likely that the absence of HHV-6-DNA in patient CSF or serum samples doesn't necessarily exclude an ongoing or recently activated HHV-6 infection. In primary HHV-6 infections the DNA load in the CSF is significantly lower than in HHV-6 reactivations, and the pathological mechanisms are thought to be different; in reactivation, virus replication in the CNS is more active and in primary infection the inflammatory mediators are more involved than straight virus replication (Kawamura et al., 2011). It may therefore be useful to perform an assay for the detection of HHV-6 antibodies if no HHV-6 DNA is found in the CSF.

HHV-6 seems to be present in early MS but association does not necessarily mean causation. It is possible that HHV-6 primary infection or activation is a consequence rather than a cause in MS disease. One hypothesis is that in MS, patients have an overactive immune system that leads to polyclonal B cell activation and it explains immune reactivity to many viruses (Dhib-Jalbut et al., 1990). Polyclonal antibody production may then be seen in the presence of HHV-6 antibodies, as well as other viral antibodies. However, in one study, MS patients demonstrated high immune reactivity to HHV-6 even when compared to other auto-immune diseases (Friedman et al., 1999). This suggests that a pathogenetic role is possible. Moreover, HHV-6 is able to reactivate in MS patients treated with natalizumab (Yao et al., 2008) and the active HHV-6 replication can decrease the response to certain MS treatments (Garcia-Montojo et al., 2011). Therefore, although HHV-6 is hardly the main agent in MS immunopathogenesis, it has to be taken into account in the evaluation of patients with suspected MS and in the treatment decisions and follow-up of patients with MS.

## 5.2 VIRUS-REACTIVE OCBs IN MS

Altogether 26 patients (out of 79) were found to carry HHV-6A and/or HHV-6B and/or VZV –reactive OCBs in their CSF near the event of clinical neurological symptoms that lead to a suspicion of a demyelinating disease. Most of the patients were soon diagnosed with MS (62%, n=16).

HHV-6 reactive OCBs have also been reported in a different MS patient cohort using the same method (Virtanen et al., 2014) and also in another MS cohort using a different method (Alenda et al., 2014).

Patients with HHV-6 or VZV-reactive OCBs seem to differ statistically in laboratory parameters from the patients who have no virus-reactive OCBs in their CSF. They have a significantly higher IgG index and more OCBs in their CSF. Patients with virus-reactive OCBs also have lower protein concentration in their CSF but this finding was not statistically significant. They were also younger than the patients with no virus-reactive OCBs. Patients with MS and HHV-6 infection have been noted to be younger in previous studies as well (Knox et al., 2000).

No significant differences were found in Barkhof's criteria among patients with or without virus-reactive OCBs. The OCB status has earlier been associated with different MRI findings that are seen in different lesion patterns but not in differences in Barkhof's criteria (Huttner et al., 2009). Differences in MRI findings have been reported in patients with virus-reactive OCBs in earlier studies as well (Virtanen et al., 2014).

No significant differences were seen in the disease courses, durations, or clinical symptoms when comparing patients with HHV-6 -or VZV -reactive OCBs to patients without reactive OCBs. MS disease course heterogeneity and the small number of patients in this study provide a challenge to find significant clinical differences between the groups of patients.

Only a portion of the patients with MS seemed to have some of the viruses, including HHV-6, involved in their disease and many patients had no signs of any viruses. It is therefore possible that the virus-reactive OCB status may simply be just be a part of a polyclonal activation and be a sign of an overly activated immune system.

Interestingly, all the differences in the CSF parameters of the patients, in these separate studies, seemed to be parallel; patients with HHV-6 -or VZV-reactive OCBs had more OCBs, higher IgG index and lower protein concentration in their CSF, and they all were also younger than the patients who

had no virus-reactive OCBs at all. The parameters that gave significant results were, however, diverse in different studies (Table 7). The number of the patients in our study is too low for a decent comparison of statistical differences: Only clear differences may give a significant result and some of the true differences may lack statistical significance.

The results seem to support the theory that patients who have signs of HHV-6 or VZV -infections seem to differ from the patients with no recent HHV-6 or VZV -infection. They seem to have signs of a possibly more aggressive intrathecal immunoglobulin synthesis with higher IgG index and more OCBs. They also seem to have active intrathecal HHV-6 or VZV antibody synthesis. This may support the theory that patients who have HHV-6 or VZV signs in their early MS may have these viruses somehow associated in their disease pathogenesis. Alternatively, there is no causative role for the viruses, but these patients who have immunologically more active disease may just be more prone to polyclonal activation of viral antibody production.

It is reported that certain MS medications, that modulate the immune system, are able to induce difficult VZV reactivations in some patients, in addition to HHV-6 reactivations (Perez-Cesari et al., 2005). It may be that activation of the virus, when the immune system is suppressed, somehow associates either by itself or by inducing polyclonal activation to the MS process. If so, these patients are at higher risk for the CNS complications that these viruses may induce. This would be noteworthy while treating these patients.

#### *5.2.1 Total number of OCB*

In many cases, patients with MS have a stable OCBs status and OCB banding pattern that has even been defined to be a unique “fingerprint” for each patient (Confavreux et al., 1986). The number of the bands may, however, change with time, also depending on the activity of the MS disease and on the effective treatments (von Glehn et al., 2012, Harrer et al., 2013, Axelsson et al., 2013, Mancuso et al., 2014, Thompson et al., 1983, Haertle et al., 2014). The same pattern is also evident with corticosteroid treatment in other autoimmune diseases with neurological involvement (McLean et al., 1995).

Patients with MS disease also have differences in their disease characteristics depending on whether they have OCBs in the CSF, and if a patient has 10 or more OCBs it is even more predictive of MS (Bourahoui et al., 2004). Therefore, it seems likely that the OCBs and B cell clusters in the CSF have

a relevant role in MS pathogenesis (Bankoti et al., 2014). In addition, drugs that reduce B cells are effective in MS treatment. The number of OCBs, however, doesn't seem to have direct correlation with the amount of active B cell clones (Petzold, 2013).

It is still possible that the higher number of bands in the CSF and the intensity of the color and band thickness mean that the amount of antibodies in the CSF is higher and it may be caused by the more active production of antibodies in the CNS. It is a sign of immune system activation in the CSF. It doesn't seem to correlate with the clinical course but it may be indicative of a difference in pathogenetic mechanisms. This is in parallel with the current study's finding that patients with virus-reactive OCBs have a higher number of OCBs. Patients with virus-reactive OCBs may have a different pathogenesis with a virus being involved. Alternatively, they may represent a subgroup of patients that are prone to mount more vivid immune reactions to external antigens.

Only one CSF sample was available from each of the patients. In most cases it was taken near or during the time of their first clinically noted symptoms because a control lumbar puncture sampling is usually not clinically justified. Thus, the number of OCBs in the CSF of the patients cannot be compared to determine whether this differed during the disease course. In addition, the number of HHV-6 –or VZV –reactive OCBs could not be compared to determine whether they are stable or if they change during the disease course.

One major weakness in our study is that we have a small number of patients from whom CSF and serum samples were analyzed. The small number of cases could possibly lead to inaccurate conclusions. The low number of patients also makes statistical analysis unreliable in finding all possible differences, especially in clinical course. Lumbar puncture is an invasive procedure which is not recommended unless necessary. These CSF samples were, however, collected systematically from all patients that were treated in one hospital within a year. According to the clinic protocol, every patient with a relevant suspicion of MS underwent lumbar puncture sampling. Therefore, even though the number of cases is low, the population of the study is rather representative of patients in the early phase of a demyelinating disease.

### *5.2.2 How can the small number of virus-reactive OCBs be explained, when compared to the total number of OCBs?*

The resolution of separate bands and the clearness of colors in the original IEF gel are clearly better when compared to the bands in the nitrocellulose membrane after affinity-driven immune blotting and immunoperoxidase staining. Some of the thickest bands in the membranes may contain several bands that would be seen as separate bands in the gel. In affinity-driven immune blotting, some of the antibodies in the bands can fail to transfer into the membrane and, therefore, don't appear in the membrane. In the IEF gels, the OCBs are often located in a broad pH range (Figure 8). In the nitrocellulose membrane, the HHV-6-reactive bands were located only in a certain part of the banding pattern. VZV-reactive OCBs were also located only in certain part of the pattern. It seems that in addition to the HHV-6 or VZV-reactive OCBs, there are additional bands which are reactive with other antigens than HHV-6 or VZV. Three patients had both HHV-6 reactive OCBs and VZV-reactive OCBs visible in the same CSF sample. IEF runs with different virus-antigens were performed separately in different runs. No markers were used that would enable locating individual OCBs and, therefore, comparison based on the location of individual bands in different runs is not easy. In these cases, the VZV-reactive and HHV-6- reactive bands were located in clearly different parts of the pattern, which makes the identification of these bands reliable. These patients truly seem to have both HHV-6- and VZV-reactive -OCBs at the same time. It may be a true immune reaction against the two pathogens or just a secondary unnecessary antibody production of B cells caused by activated immune system (polyclonal activation). There are several reports of co-infections especially involving herpes viruses (Gaeta et al., 2009, Al Fawaz et al., 2014, Mancuso et al., 2007). Co-expression of HHV-6 and VZV is also seen in the ability of these viruses to be latent in the same site at the same time (Hufner et al., 2007). Co-existence of HHV-6 and EBV DNA has also been associated with RRMS disease activity (Hollsberg et al., 2005). The patients seemed to have a clear timely relation with virus-reactive OCBs and their clinical symptoms (Figure 9) but the meaning of this finding requires more research.

### *5.2.3 Virus-reactive OCBs; polyclonal activation or antibody production against relevant antigens?*

The presence of antibodies against measles and/or rubella and/or varicella (MRZ-reaction) in CSF is considered to be a highly typical characteristic in MS and it is considered to predict CIS progression into MS as well (Bednarova et al., 2005, Stangel et al., 2013, Jarius et al., 2008, Brettschneider et al.,

2009, Stich et al., 2014). MRZ-reaction is thought to be a sign of polyclonal immune activation in an immune system dysfunction (Skorstad et al., 2009, Kulakowska et al., 2012). These reports suggest that virus-specific OCBs in the CSF are not produced against its own target and are not a sign of an infectious agent. Some reports suggest that HHV-6 antibodies are a part of a polyclonal immune response rather than a causative agent in MS (Derfuss et al., 2005).

Besides relevant antibody production, virus infections may also trigger abnormal B cell cytokine responses (Bar-Or et al., 2010). This combination is probably seen in the high amount of IgG in the CSF and as a high number of OCBs. It has also been considered that MS is rather triggered by multiple infectious agents together than antibodies being just a product of a polyclonal activation (Krone et al., 2008). Antigen-driven immune response seems to dominate in MS pathogenesis (Gilden, 2005) and B cells are natural target cells in the different therapies for MS (Krumbholz et al., 2012).

### 5.3 MECHANISMS FOR HOW HHV-6 AND VZV COULD BE INVOLVED IN DEMYELINATION AND MS PATHOGENESIS

The association of HHV-6 and VZV with the MS pathogenesis remains controversial. Both viruses are found in the early or active phase of the MS disease. Both viruses also seem to fit in to the viral theory of MS, especially HHV-6. It therefore seems plausible that HHV-6 and VZV could have some role in the disease progression.

#### 5.3.1 *Cell-mediated and humoral autoimmunity theories of MS*

##### 5.3.1.1 Molecular mimicry

MBP-reactive T cells can be activated by HHV-6 through molecular mimicry because HHV-6 shares some identical sequence with MBP, which the T cells recognize (Tejada-Simon et al., 2003, Tait and Straus, 2008). After cross-reactivation, the T cells attack against the host myelin. This results in demyelination.

##### 5.3.1.2 Interaction with leukocytes

HHV-6 may activate CD4<sup>+</sup> T-cells via its interaction with CD46 (Yao et al., 2010b). CD46 is a membrane co-factor protein that is one of the regulators in a complement cascade. CD46 is also a

cellular receptor for HHV-6A (Santoro et al., 1999). Increased levels of CD46 and HHV-6 infection have been reported in patients with MS (Fogdell-Hahn et al., 2005, Alvarez-Lafuente et al., 2009). Especially CD46 is associated with HHV-6A virions (Hammarstedt et al., 2007). HHV-6 may even induce leukocytes to travel into the CNS without producing an infection (Reynaud et al., 2014).

HHV-6B uses the CD134 as a cellular receptor. CD134s are mainly located on activated T cells, but not on glial cells. (Tang et al., 2013)

Invariant natural-killer T cells are one of the controls of VZV in human hosts and it is thought that abnormalities in these cells lead to VZV reactivation (Novakova et al., 2011). VZV can cause a non-productive infection in neurons and prevent infected neurons to undergo apoptosis (Pugazhenthil et al., 2011, Yu et al., 2013). It may also have an impact on the immune system and lead to over-or under-regulation of the system.

### 5.3.2 *Direct virus infection of the CNS*

HHV-6A uses CD46 as a cellular receptor and enters into the target cell via the cellular-membrane lipid raft (Tang et al., 2008). CD46 is a highly expressed protein in the BBB, and it is thought that HHV-6 could use CD46 for passing the BBB into the CNS (Alvarez-Lafuente et al., 2009, Shusta et al., 2002). This may be one mechanism that could explain how HHV-6A is capable of a direct CNS infection.

HHV-6 is capable of directly infecting oligodendrocytes (Ahlqvist et al., 2005). It may induce apoptosis of oligodendrocytes and neurons (Gardell et al., 2006). It may also indirectly induce death of necrotic oligodendrocytes and therefore cause demyelination (Kong et al., 2003). HHV-6 may lyse infected target cells or it may activate inflammatory or immune reactions and induce an autoimmune reaction (Krueger and Ablashi, 2003).

HHV-6 can directly infect the CNS and seroconversion may be seen until recovery (Agut, 2011). One entry mechanism is known to be the olfactory pathway (Harberts et al., 2011). One patient showed similar findings of a direct CNS infection associated with HHV-6 reactive OCBs in the CSF and no serum antibodies to either of the HHV-6 variants. Three patients with signs of a primary infection in the serum antibodies also had HHV-6-reactive OCBs present in their CSF at the same time. This supports the theory that HHV-6 is capable of infecting the CNS directly.



VZV can infect axons and retrogradely transport itself into neuronal cell bodies (Markus et al., 2011). This may be one pathway for VZVs to enter the CNS.

### *5.3.3 Gives access to other pathogens to infect the host CNS?*

HHV-6 can cause slight suppression for the human immune system and allow access to other factors (for example other viruses) to induce MS progression. This may be one possible explanation as to why some of our patients, who were diagnosed with MS, had an HHV-6-co-infection with either the other HHV-6-virus type or VZV. Co-infections of viruses have been reported previously in early MS (Ferro et al., 2012, Tomsone et al., 2001). HHV-6 has even been shown to increase the replication of JC viruses (JCV) (Yao et al., 2008).

HHV-6 may cause immunosuppression through the ability to directly infect CD4+T cells (Lusso, 2006). HHV-6 is also capable of inducing HHV-6- CD4+ and CD8+ -specific regulatory T cells that both suppress CD4+ T cells and reduce dendritic cell functions (Wang et al., 2014). HHV-6 seems to have an impact on the cytokine network. It is capable of modulating the synthesis of different cytokines, which leads to immunosuppressive effects for different cell types (i.e. macrophages, astrocytes, leukocytes) (Chapenko et al., 2003, Meeuwssen et al., 2005, Tejada-Simon et al., 2002, Gustafsson et al., 2013b).

## 6 CONCLUSIONS AND FUTURE ASPECTS

HHV-6 has been connected with MS progression in many studies, especially in the relapsing-remitting type of the disease. Some studies dispute that HHV-6 is involved in the disease pathogenesis and the relation between HHV-6 and MS still remains at least partly controversial.

In early MS and in other related demyelinating diseases of some patients, HHV-6 is seen as a serological primary infection and also in the CSF as HHV-6- reactive OCBs. It seems to play a role in the disease progression, at least in some patients. In immune competent adults, HHV-6 seems to have no typical clinical signs of a virus infection.

In this study, differences between the patients who had VZV-reactive OCBs and the patients who had no VZV-reactive OCBs were similar to the case with HHV-6-reactive OCBs. The patients who had either VZV or HHV-6- reactive OCBs in their CSF had a statistically higher number of OCBs, higher IgG index, lower protein concentration, and they were younger than the patients who had no reactive OCBs. Significant differences were observed in the different parameters within VZV and HHV-6 OCB-reactive patients, but all the differences showed a similar trend. The small number of patients prevents detailed statistical analyses and definite conclusions. The results, however, support the theory of the association of HHV-6 and VZV with the disease pathogenesis and that they may even be triggering the MS progression in some patients.

It seems that there are distinguishable subgroups formed by different viruses in the diseases with demyelination, and this can be seen as the presence of virus-reactive OCBs. It is likely that no single virus will be found to trigger MS, but more likely that many different viruses will be found to be involved in different “subgroups” of the disease course, as it seems to be with HHV-6. The diagnosis of HHV-6 and other herpes virus infections should be implemented in the differential diagnostic process to be able to treat herpesvirus induced neurological symptoms.

There are antiviral drugs that are relatively safe and known to be effective in HHV-6 infections. Studies of antiviral therapy treatment against HHV-6 and its effect to the course of demyelination could give useful further information. The search for HHV-6 infection is relevant in the follow-up of MS patients who are on immunosuppressive therapies and hence at higher risk for the CNS complications that these viruses may induce.

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SUPPLEMENTS

Potilaskaavake

Potilas						
Sukupuoli M / N						
Ikä						
Likvori	solut	prot.	gluk.	eryt.	leuk	
	IgG index	oligoklon.	ACE	lysots.	borrelia IgG	borrelia IgM
	virusseula (mikä koholla)			Borr NhO		
Seerumi	borrelia	ACE	TPHA	(/kardioliini)		
MRI						
	1 muutos	2-4 muutosta		>4 muutosta		
	pään:					
selkäytimen:	muutoksia	ei muutoksia		mahd. demyelinaatio		
herätevaste						
BAEP:	ei tehty	vas. +	vas. norm	oik. +	oik. norm	

VEP:	ei tehty	vas. +	vas. norm	oik. +	oik. norm
SEP:	ei tehty	vas. +	vas. norm	oik. +	oik. norm
Kliiniset oireet	alkanut: (kk/v)	oireet: (+vaikeusaste, tukevia statuslöydöksiä)			
shuubi 1:					
shuubi 2:					
kortisonipulssi/ jättikortisoni	ei saanut	saanut	pvä		
Muut sairaudet:					